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Liquid chromatography–tandem mass spectrometry analysis of cocaine and its metabolites from blood, amniotic fluid, placental and fetal tissues: study of the metabolism and distribution of cocaine in pregnant rats

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Abstract

The ability to simultaneously quantitate cocaine and its 12 metabolites from pregnant rat blood, amniotic fluid, placental and fetal tissue homogenates aids in elucidating the metabolism and distribution of cocaine. An efficient extraction method was developed to simultaneously recover these 13 components using underivatized silica solid-phase extraction (SPE) cartridges. The overall recoveries for cocaine and its metabolites were studied from pregnant rat blood (47–100%), amniotic fluid (61–100%), placental homogenate (31–83%), and fetal homogenate (39–87%). Extraction of the samples using silica is not classical SPE, but rather allows for the concentration of the sample into a small volume prior to injection and the removal of the proteins due to their strong interaction with the active silica surface. A positive ion mode electrospray ionization liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was used and validated to simultaneously quantitate cocaine and 12 metabolites from these four biological matrices. A gradient elution method with a Zorbax XDB C_8 reversed-phase column was used to separate the components. Multiple reaction monitoring (MRM) of a product ion arising from the corresponding precursor ion was used in order to enhance the selectivity and sensitivity of the method. Low background noise was observed from the complex biological matrices due to efficient SPE and the selectivity of the MRM mode. Linear calibration curves were generated from 0.01 to 2.50 ppm. The method also showed high intra-day ($n=3$) and inter-day ($n=9$) precision (% RSD) and accuracy (% error) for all components. The limits of detection (LODs) for the method ranged from 0.15 to 10 ppb. The LODs of cocaine and its major metabolites were less than 1 ppb from all four biological matrices. This method was applied to the study of the metabolism and distribution of cocaine in pregnant rats following intravenous infusion to a steady state plasma drug concentration. The following results were observed in the pregnant rat study: (1) the observations correlated strongly with the previous literature data on cocaine metabolism and distribution, (2) cocaine and norcocaine accumulated in the placenta, (3) arylhydroxylation of cocaine was a major metabolic pathway, (4) *para*-arylhydroxylation of cocaine was favored over *meta*-arylhydroxylation in rats and (5) accumulation of cocaine and its major metabolites was observed in the amniotic fluid.

Keywords: Cocaine; Cocaine, metabolites

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1. Introduction

Cocaine, the major alkaloid of *Erythroxylum coca*, has been widely abused since its existence and effects were known to man. As in the case of most drugs of abuse, cocaine exposure causes feelings of euphoria and well being but subsequently leads to a ''crash'' with depression and discomfort. After entering the central nervous system, cocaine induces release of neurotransmitters producing this feeling of euphoria [1–7]. In addition, cocaine also blocks the re-uptake of neurotransmitters after their release by nerve cells, extending this effect. The subsequent depletion of neurotransmitters such as dopamine causes this ''crash''. Other effects of cocaine include an increase in heart rate, blood pressure, and pupil diameter. Cocaine use has also been reported to cause chronic damage to the cardiovascular and nervous systems [8–11].

The use of cocaine by pregnant women has become a public health concern. It is estimated that, in the United States, 2 to 3% of women abuse cocaine during pregnancy [12]. Neonates born to cocaine using women may experience complications such as low birth weight, preterm delivery, fetal malformations, stillbirths, and neurodevelopmental abnormalities [13]. The incidence of delivery-related complications for cocaine exposed infants climbs dramatically if the mother has abused other substances such as alcohol, caffeine, nicotine or controlled substances [13,14]. The exact nature of these interactions is not known but may be related to altered metabolism and placental transport of cocaine [15–21]. The use of cocaine during pregnancy is associated with a 200–300% increase in the cost of delivery of these ''crack babies'' [22,23]. The cost to the US healthcare system is approximately US$1 billion annually [22]. This situation is exacerbated by the lack of complete understanding of metabolism and distribution of cocaine during pregnancy. The ultimate goal of this effort is to study cocaine fetal uptake, metabolism and disposition. One of the difficulties in studying cocaine transport and metabolism in the fetus has been the lack of animal models. The first step in the study of cocaine metabolism and distribution was identified as the development of animal models that closely mimic the human system. The pregnant rat model has been previously used to study the transport of substances from the maternal to fetal compartment [5,15,24]. In this study, we have developed and validated a method to measure levels of cocaine and 12 metabolites (ecgonine methyl ester, ecgonine ethyl ester, benzoylnorecgonine, ecgonine, benzoylecgonine, norcocaine, *p*-hydroxycocaine, *m*-hydroxycocaine, *p*-hydroxybenzoylecgonine, anhydroecgonine methyl ester, cocaethylene, norcocaethylene) from maternal blood, amniotic fluid, placental and whole fetal homogenate. This method was then used to determine the metabolism and distribution of cocaine in pregnant rats.

The analysis of cocaine and its metabolites presents many analytical challenges. Cocaine is rapidly metabolized in vivo and therefore does not provide a long window for detection. The simultaneous analysis of cocaine and its metabolites also presents difficulties in the extraction from biological matrices and chromatographic separation because of the large polarity difference between the non-polar cocaine and its highly polar metabolites.

The determination of cocaine and its metabolites from various matrices such as plasma [25–27], urine [28,29], hair [26,30,31] and saliva [32] has been reported. More recently, meconium, the first stool pass of a neonate, has attracted attention since it may reveal the history of maternal cocaine use during the second and third trimesters of pregnancy [28,29,33–39]. Currently, most cocaine analysis is done by gas chromatography–mass spectrometry (GC–MS) [40–42]. In GC–MS studies, cocaine metabolites containing hydroxyl or carboxyl groups need to be derivatized as fluoroesters or silanoesters to mask the polar, hydrogen bonding characteristics of the molecule. The derivatization agents usually are pentafluoropropionic anhydride (PFPA), bis-(trimethylsilyl)trifluoroacetamide (BSTFA) or trimethylchlorosilane (TMCS). The limit of quantitation of cocaine in GC–MS methods is generally around 10 ppb. In order to minimize sample handling, techniques that do not require derivatization, such as high-performance liquid chromatography (HPLC) have recently gained in popularity [43–46]. Many of the polar metabolites of cocaine possess little inherent UV absorption limiting the usefulness of UV detection when coupled with HPLC thereby making MS an attractive choice for this type of

analysis. However, most LC–MS [46] and LC–MS–MS methods [27,47,48] to date have focussed on only a few metabolites of cocaine. While many of the minor metabolites are inactive, several of them possess significant pharmacological/toxicological activity and therefore merit monitoring [44]. In addition, many newly discovered metabolites have unknown pharmacological/toxicological activity. The general metabolic pathways of cocaine are shown in Fig. 1.

Benzoylecgonine is one of the major metabolites formed by either spontaneous hydrolysis or a hepatic carboxyesterase. The other major metabolite is ecgonine methyl ester, which is formed by hepatic cholinesterase. Both of these metabolites have great utility in the detection of cocaine exposure, due to their long half-lives in biological matrices (approximately five times longer than cocaine). Both benzoylecgonine and ecgonine methyl ester are further hydrolyzed to the remaining major metabolite, ecgonine.

N-Demethylation of cocaine to norcocaine has been identified as a minor pathway in humans. However, norcocaine is a metabolite of cocaine found to have significant in vivo pharmacological activity (reported as five-times as potent as cocaine itself). Several metabolites of norcocaine, such as *N*-hydroxynorcocaine and norcocaine *N*-oxide have been suggested as mediators of cocaine's hepatic toxicity in humans [49]. Norcocaine can also be hydrolyzed to benzoylnorecgonine, a metabolite that is known to cause seizures in rats [50]. Cocaine on co-administration with alcohol leads to the formation of cocaethylene, large concentrations of which are found in the urine of pregnant cocaine users suggesting that accumulation in the human fetus is likely [51].

Small amounts of several arylhydroxy metabolites of cocaine have been identified in the urine of adult cocaine users and in the meconium and urine of neonates [28]. These metabolites include *m*- and *p*-hydroxybenzoylecgonine and *m*- and *p*-hydroxycocaine. Of these, *m*-hydroxybenzoylecgonine has drawn the most attention due to its cross-reactivity

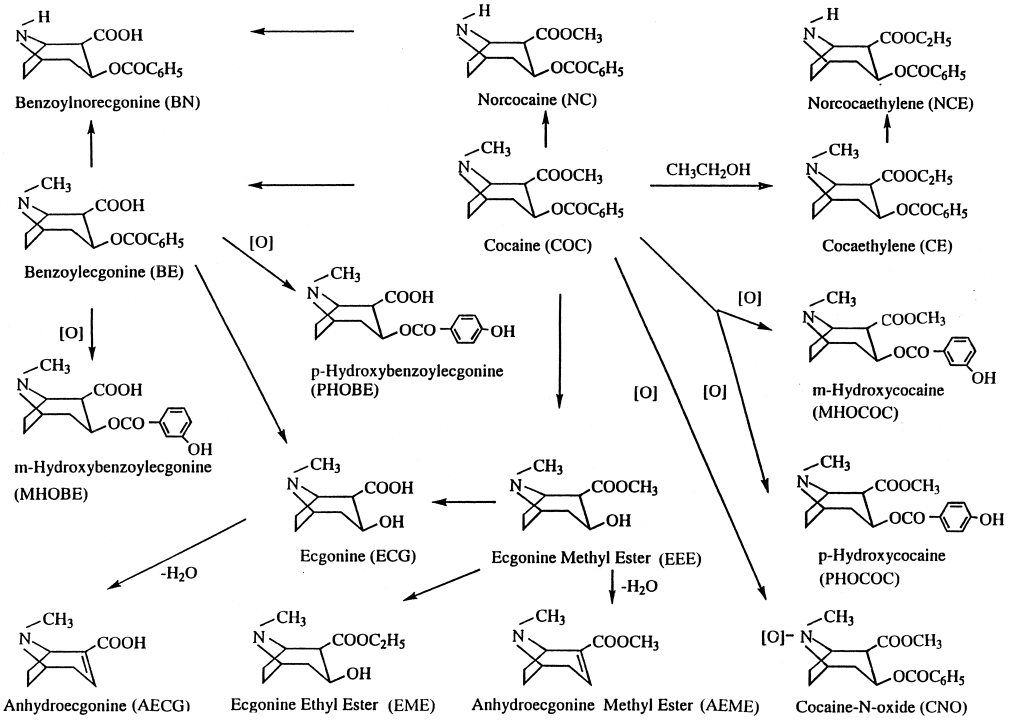

Fig. 1. Structures of cocaine and its metabolites and general pathways of cocaine metabolism.

with the benzoylecgonine enzyme immunoassay (EIA) [52]. The pharmacological activity of these arylhydroxycocaine metabolites is presently not known. In rats the level of *m*-hydroxybenzoylecgonine was found to be low and near the limit of detection (LOD) and therefore was not included in the assay.

In this study, a highly sensitive LC–MS–MS method was developed and validated to determine the concentrations of cocaine and 12 metabolites. The levels of cocaine and its metabolites were determined from blood, amniotic fluid, placental and fetal tissues of a pregnant rat after extraction. Cocaine was then infused intravenously into a pregnant rat to a steady state plasma drug concentration and the levels of cocaine and its metabolites were measured. This provided information on cocaine metabolism and distribution in various compartments. The results correlated with previously reported literature data on cocaine in vivo metabolism and distribution making this a reliable assay for the development of animal models for cocaine studies.

2. Experimental

2.1. Chemicals

(−)-Cocaine hydrochloride (COC), (−)-ecgonine methyl ester hydrochloride (EME), (−)-ecgonine ethyl ester hydrochloride (EEE), (−)-benzoylnorecgonine hydrochloride (BN), (−)-ecgonine hydrochloride (ECG), (−)-benzoylecgonine (BE), (−)-*N*-norcocaine (NC), [N-C^2H_3]cocaine hydrochloride (D_3-COC), [N-C^2H_3]benzoylecgonine (D_3-BE), and [N-C^2H_3]ecgonine methyl ester hydrochloride (D_3-EME), anhydroecgonine methyl ester (AEME), cocaethylene (CE) and norcocaethylene (NCE) were provided by the National Institute on Drug Abuse (Rockville, MD, USA). *p*-Hydroxycocaine (PHOCOC), *m*-hydroxycocaine (MHOCOC), *p*-hydroxybenzoylecgonine (PHOBE) and *m*-hydroxybenzoylecgonine (MHOBE) were purchased from Research Biochemicals International (Natick, MA, USA). The structures of cocaine and its metabolites are shown in Fig. 1.

Methanol and acetonitrile (both HPLC-grade), formic acid (88%) and glacial acetic acid (J.T. Baker, Philipsburg, NJ, USA) and ammonium acetate (97+%, Aldrich, Milwaukee, WI, USA), were used without further purification. Deionized water was generated from a Continental Deionized water system (Natick, MA, USA).

2.2. Instrumentation

HPLC separations of samples were achieved on a Hewlett-Packard (Palo Alto, CA, USA) Model 1100 system. Several columns were tested to achieve optimal separation and sensitivity. Among these columns, two appeared to be superior to the others. These were the Zorbax 300 SB-C_{18} column (150 mm×2.1 mm, 5 μm) and Eclipse XDB-C_8 column (150 mm×2.1 mm, 5 μm) which were obtained from MAC-MOD Analytical (Chadds Ford, PA, USA). Column temperature was maintained at 37°C during a run and the column flow-rate was 0.27 ml/min. The mobile phases used in this study were (A) 2.5 m*M* ammonium acetate (pH 2.7)–acetonitrile (96.75:3.25); (B) methanol–acetonitrile (50:50); and (C) 20 m*M* ammonium acetate (pH 2.7). The gradient tables for both the C_{18} and C_8 columns are shown in Table 1.

Mass spectrometric experiments were performed using a Micromass Quattro II (Beverly, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source heated to

Table 1
Gradient tables for separation of cocaine and its metabolites using (A) C_{18} and (B) C_8 columns

Column	Mobile phase		Time (min)
	A	B	
C_{18}	95	5	0
	95	5	2
	85	15	7
	85	15	15
	0	100	23
C_8	C	B	
	100	0	0
	100	0	2
	85	15	7
	85	15	15
	0	100	23

120°C. The capillary and cone voltages were optimized to 3.5 kV and 35 V, respectively. The analytical quadrupoles were set to unit mass resolution. At the first stage of method development, both LC–MS and LC–MS–MS were explored. In LC–MS experiments, the selected ion recording (SIR) mode was employed. In SIR, each individual m/z value was monitored and integrated. SIR acquisitions are used in the detection of known or targeted compounds to give enhanced sensitivity above full scan acquisitions. In LC–MS–MS experiment, the multiple reaction monitoring (MRM) mode was employed. MRM monitors the pathway of a selected precursor ion to a selected fragment ion upon collision induced dissociation. Before the MRM mode was used, flow injection MS–MS spectra of the individual compounds of interest were studied to decipher their collision induced dissociation patterns, identify the most intense fragment ions, and obtain the optimized collision energies and other instrumental parameters.

Based upon the criteria of Matuszewski et al. [53], the biological matrices (blood, amniotic fluid, placental and fetal homogenates) did not present interference or create ion suppression of the analytes of interest in the MRM mode. Matuszewski et al. suggested that ion suppression due to co-eluting matrix components could be greatly reduced by an efficient chromatographic separation (with high analyte retention) in addition to an efficient and selective extraction method. By increasing the retention factor (k') they observed practical elimination of ion suppression due to separation of matrix components from the analytes [53]. The chromatographic method developed for cocaine and its metabolites shows high selectivity and high analyte retention of the components thereby aiding in the reduction of matrix component related ion suppression effects. In addition, Matuszewski et al. studied the intensities of analyte peaks from biological matrices obtained from multiple sources [53]. They also suggested that the slopes of standard curves obtained from these different matrices would indicate matrix related ion suppression effects [53]. In this study, matrices from different rats were analyzed and peak intensities and slopes of the standard curves were found not to vary substantially (relative standard deviation, RSD$<$ 10%), indicating the absence of matrix related ion suppression effects. The solid-phase extraction (SPE) method used in this method was very specific for the recovery of basic compounds in the matrix (as described Section 3.4) and hence supports elimination of components in the matrix that may lead to analyte ion suppression.

The MRM mode proved to be more sensitive because of lower levels of background related noise when compared to the SIR mode. Therefore, the quantitation work reported in this article was performed using the MRM mode and LC–MS–MS. Thirteen channels (16 components) with the following precursor m/z→product m/z values were selected and monitored using a 0.2 m/z span and a 0.02 s inter-channel delay: 186.2→168.1 (ECG), 200.3→182.2 (EME), 214.2→196.3 (EEE), 203.3→185.1 (D_3-EME), 182.2→118.2 (AEME), 276.3→154.1 (BN), 290.3→168.0 (BE, NC), 293.3→171.2 (D_3-BE), 304.2→182.2 (COC, NCE), 306.2→168.1 (PHOBE), 307.2→185.1 (D_3-COC), 320.2→182.2 (PHOCOC, MHOCOC) and 318.3→196.2 (CE) [54].

2.3. Sampling

Sprague–Dawley rats were maintained on a 12-h light/dark cycle at 22°C and allowed food (standard laboratory chow) and water ad libitum. All animal work was conducted under approval of the University of Georgia Animal Use and Care Committee. At least 4 days before the experiment, the rat arrived at the ALAAC approved facility to acclimate. On the morning of the experiment (on day 19 of pregnancy), the rat was moved into the laboratory and anesthetized using a ketamine–acepromazine (50:3.3 mg/kg) solution. Catheters were placed in the right jugular and left femoral veins. A loading dose of cocaine was administered intravenously into the rat at a concentration of 5 mg/kg of body weight of the rat. Since the average body weight of the pregnant rats was 304±24 g, a loading dose of approximately 1.5 mg was administered. Cocaine was infused intravenously after the loading dose at the rate of 90 μg/min at a flow-rate of 30 μl/min. Cocaine was infused for 1 h before sampling to assure that steady state levels for cocaine were achieved.

After 1 h, serial 400-μl samples of blood were collected into chilled heparinized tubes containing

D_3-COC internal standard solution (40 μl of 2 ppm solution). To each tube, 40 μl of 0.64 *M* sodium fluoride solution was added. The tubes were vortex-mixed and centrifuged immediately. Sodium fluoride (NaF) was added to act as the inhibitor for esterases thereby preventing cocaine hydrolysis. After centrifuging the plasma was transferred to a clean chilled tube and flash frozen with liquid nitrogen in order to minimize the degradation of the analytes. Amniotic fluid samples (400 μl) were collected in chilled tubes with the NaF inhibitor and the internal standard (D_3-COC, 40 μl of 2 ppm solution) and flash frozen prior to storage. The placental and fetal tissues were rapidly removed, and immediately rinsed with ice-cold water to wash off excess blood and to cool the tissue. The tissues were then blotted dry, weighted and finely minced with scissors. 60 μ*M* NaF was the added to the minced tissues before they were homogenized. The volume of NaF added was twice the weight of the corresponding tissue sample. Internal standard (D_3-COC, 40 μl of 2 ppm solution) was added to 400 μl of the tissue homogenates. Samples were then stored at −20°C until analysis (2–5 days total). The above plasma, amniotic fluid, placental and fetal homogenate samples were centrifuged and the supernatants were then extracted using SPE.

2.4. Preparation of calibrations standards for SPE

Six standard solutions were prepared for spiking on biological matrices for generation of calibration curves. The concentrations of these solutions ranged from 0.01 ppm to 2.50 ppm of cocaine and its 12 metabolites. A 400-μl volume of each of these six standard solutions and 40 μl of 2.0 ppm of the internal standard, D_3-COC were added to tubes and evaporated under vacuum (SC110A SpeedVac Plus and RVT400 refrigerated vapor trap, Savant, Farmingdale, NY, USA). Four sets of such evaporated standards, one set for each of the four biological matrices were prepared. A 400-μl volume of blank blood was collected into the first set of tubes. To these tubes, 40 μl of 0.64 *M* NaF solution was added. The tubes were vortex-mixed and centrifuged immediately. NaF was added to act as the inhibitor for esterases thereby preventing cocaine hydrolysis. After centrifuging, the plasma was transferred to a clean chilled tube and flash frozen with liquid nitrogen in order to minimize the degradation of the analytes. A 400-μl volume of blank amniotic fluid was then added to the second set of standards, vortex-mixed and then centrifuged. The supernatant was then used for generation of calibration curves. For generation of calibration curves from tissue samples, the blank tissues were rapidly removed, and immediately rinsed with ice-cold water to wash off excess blood and to cool the tissue. The tissues were then blotted dry, weighted and finely minced with scissors. 60 μ*M* NaF was the added to the minced tissues before they were homogenized. The volume of NaF added was twice the weight of the corresponding tissue sample. A 400-μl volume of homogenized placental and fetal tissues in sodium fluoride was added to the next two sets of tubes containing standards, vortex-mixed and then centrifuged at 10 000 rpm for 30 min. The supernatants were then used for extraction. In order to study the inter-day precision and accuracy of the method, unknowns at low (0.25 ppm) and high concentrations (1.00 ppm) of cocaine and its metabolites were prepared as described above from all the four biological matrices. Their concentrations were measured using the calibration curves made using the standard samples prepared earlier. This method is described in the following sections.

2.5. Extraction procedure

The extraction of cocaine and its metabolites was performed using underivatized silica SPE cartridges. The final extraction procedure that was optimized after evaluating different extraction procedures is as follows. The cartridge was first conditioned using 2 ml of methanol followed by 2 ml of deionized water. Secondly, the standards at the six calibration concentrations, the two unknown concentrations (prepared by spiking cocaine and its metabolites on to biological matrices as described in the previous section) and the real pregnant rat samples were loaded and drawn through the cartridge using low vacuum (~ 5 in.Hg; 1 in.Hg=388.638 Pa). After discarding the eluent, analytes in the cartridge were eluted using 3 ml of 5% ammonia in methanol solution into a clean culture tube. A washing step is useful in removing interferences in the biological

matrices that may affect the assay. This step was not performed in this extraction method because of the unacceptable loss in recoveries of highly polar metabolites such as ecgonine and ecgonine methyl ester during the washing step. However, the high selectivity and low observed ion suppression with this method makes the lack of this washing step less of a concern. The eluents from the extraction cartridge were then dried using a vacuum centrifuge. The residue was reconstituted in 0.4 ml of deionized water. A 100-μl volume of this solution was then taken for LC–MS–MS analysis. Recoveries were determined using a 1 ppm solution of each of the components of interest spiked into the biological matrices. The ratio of the relative area of the components of interest from the extracted fluids and tissue standard and the water solution were used to represent the recoveries.

2.6. Generation of calibration curves

Calibration curves for cocaine and its metabolites were generated by using the extraction method described in the previous section. Silica SPE cartridges were used because they provided extracts of high quality as described in Section 3.4. The samples were analyzed using the LC–MS–MS method described earlier. The calibration curves were plotted using the ratio of peak areas of drugs/internal standard versus that of concentrations added to the blank biological fluids. Many of the components had correlation coefficients greater than 0.99 with all metabolites showing correlation coefficients of at least 0.98. These calibration curves were generated during different days in order to study the ruggedness, inter- and intra-day precision and accuracy of the method.

3. Results and discussion

3.1. Separation with C_8 and C_{18} columns

The separation of cocaine and its metabolites was explored using numerous HPLC columns. Of these, the Eclipse C_8 and Zorbax C_{18} columns gave the highest performance in terms of analyte resolution. The respective gradient programs are shown in Table 1. Both gradient programs are the results of optimizing the following parameters: buffer concentration, pH value, gradient table and the ratio of organic modifier.

Both the C_{18} and C_8 columns achieved the separation of all components except ECG and EME, which were not completely resolved. Quantitative analysis using LC–MS–MS does not require baseline resolution of all components (such as ECG and EME) as long as the unresolved peaks have different m/z values or produce fragment ions of different masses. Baseline resolution of PHOBE and MHOBE (m/z 306), PHOCOC and MHOCOC (m/z 320) was needed due to their isomeric structures and thus identical mass-to-charge ratios.

Of the optimizable LC parameters, the pH value of the buffer was found to be the most critical. At buffer pH values greater than 3.5, a separation was not achievable. The buffer concentration could vary over the range from 10 to 100 m*M* without affecting the separation significantly. The final separation of cocaine and its metabolites using the Eclipse C_8 column is shown in Fig. 2.

3.2. Limit of detection for cocaine and its metabolites

The LODs for cocaine and its metabolites were determined by gradually reducing the concentrations of the standard solutions. To avoid possible error from sample carry-over from the previous injection, blank runs were made between each injection. For LOD studies from biological matrices, standard solutions were spiked into the matrix and extracted using the silica SPE cartridges. The measured values for the LODs for cocaine and several metabolites extracted from biological matrices are shown in Table 2. The LOD was determined as a concentration at which the signal/noise ratio was >3. The LODs of COC and its metabolites were found in the range from 0.2 ppb to 1.3 ppb in pregnant rat blood, in amniotic fluid from 0.2 ppb to 3.5 ppb, in placenta from 0.2 ppb to 5.0 ppb and in fetus from 0.2 ppb to 10.0 ppb. Cocaine and its major metabolites were found to have LODs$\leq$1 ppb in all four biological matrices.

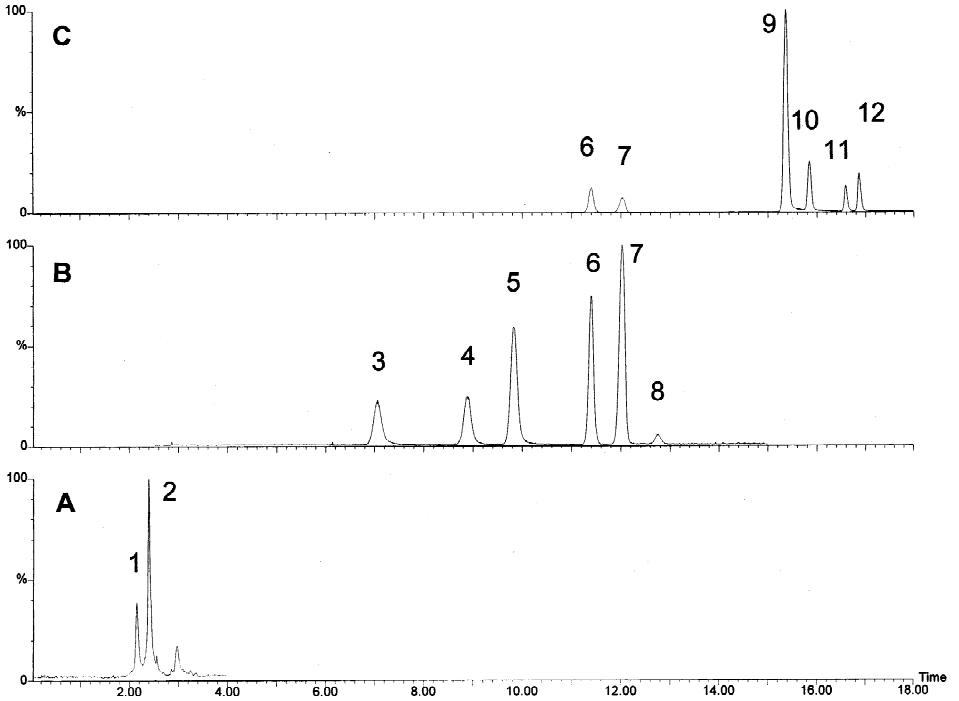

Fig. 2. ESI-LC–MS–MS total ion chromatograms from a sample of fetal homogenate spiked with cocaine and its metabolites at a concentration of 50 ppb. Panel A shows the total ion chromatogram for ecgonine (1), ecgonine methyl ester (1), anhydroecgonine methyl ester (2) and ecgonine ethyl ester (2). Panel B shows the total ion chromatogram for *p*-hydroxybenzoylecgonine (3), *m*-hydroxybenzoylecgonine (4), *p*-hydroxycocaine (5), *m*-hydroxycocaine (6), benzoylecgonine (7) and benzoylnorecgonine (8). Panel C shows the total ion chromatogram from cocaine (9), norcocaine (10), cocaethylene (11) and norcocaethylene (12).

Table 2
The limit of detection (LODs) for the ESI-LC–MS–MS method from pregnant rat blood, amniotic fluid, placenta and fetus

Analyte	LOD of cocaine and its metabolites (ppb)			
	Plasma	Amniotic fluid	Placenta	Fetus
CE	0.20	0.20	0.20	0.20
COC	0.50	0.30	0.20	0.20
NCE	0.30	0.30	0.50	0.50
NC	0.30	0.30	1.00	0.30
PHOCOC	0.30	1.00	1.00	1.00
MHOCOC	0.30	1.00	1.00	1.00
PHOBE	1.00	1.00	1.00	1.00
BE	0.50	0,50	0.50	0.20
BN	1.25	3.50	5.00	10.00
EEE	1.25	1.00	5.00	1.00
EME	0.25	0.30	0.20	0.15
ECG	1.00	1.00	1.00	1.00
AEME	1.00	1.00	1.00	0.15

3.3. Selection of internal standard for the LC–MS–MS assay

For LC–MS–MS analysis, deuterated (D_3) internal standards of the component of interest provide the most reliable and reproducible quantitative results. This is because both the internal standard and the analyte have almost identical retention times and other chromatographic properties as well as similar recoveries from biological matrices. However, not all of the metabolites of cocaine are available in deuterated form. Currently, there are five deuterated internal standards available, D_3-COC, D_3-BE, D_3-EME, D_3-NC and D_3-CE. These deuterated compounds cover the polarity range of all components of interest from highly polar (D_3-EME), moderately polar (D_3-BE) to non-polar (D_3-COC, D_3-NC, D_3-CE). The RSDs of 11 components using three different internal standards (D_3-COC, D_3-BE and D_3-EME) were calculated and compared. All of the components

showed acceptable RSD values (from 0.5% to 8%) using any one of the three deuterated compounds as an internal standard. Generally, the components eluting later tended to have a better RSD than early eluting components when using D_3-COC as the internal standard. Conversely, components eluting earlier tended to have a better RSD than the later eluting components when using D_3-EME as the internal standard [refer to the total ion current (TIC) chromatogram for the elution order]. This trend is the result of the similarity between the polarity of the components and the internal standards. For this study, D_3-COC was used as internal standard for all quantitative analysis due to its overall effectiveness and for its usefulness in monitoring ester hydrolysis during sample preparation and extraction. Evidence of ester hydrolysis would be observed from the presence of either D_3-EME or D_3-BE in the samples, which could only arise from hydrolysis of the internal standard.

3.4. The recovery of cocaine and its metabolites from matrices using various SPE cartridges

To investigate the extraction efficiency of cocaine and its metabolites out of the various biological matrices (blood, amniotic fluid, placental and fetal tissues), a variety of SPE cartridges, representing a wide-range of sorbents were used. The specific cartridges chosen for these studies were Bond Elut Certify (Varian, Harbor City, CA, USA), Oasis HLB (Waters, Milford, MA, USA), Extract-Clean silica and cyano (Alltech, Deerfield, IL, USA) cartridges. Different extraction procedures were followed for these sorbent phases in accordance with the literature methods reported for SPE using those phases. These extraction procedures were also optimized considering the polarity of the phase and the basic nature of the analytes. The recoveries of cocaine and its metabolites using the various SPE cartridges and extraction procedures were then compared to identify the most efficient SPE method. The experimental results showed that the silica cartridges had good recoveries for all the components of interest and provided the cleanest extracts. Extracts from the silica cartridges were colorless and contained no interfering peaks in the LC–MS–MS chromatograms. Extracts from the remaining SPE cartridges appeared yellowish and contained some interfering peaks in the LC trace (especially the UV trace). The recoveries were high and reproducible when using silica cartridges and the recoveries and standard deviations (SDs) for the extraction method are shown in Table 3. The HLB cartridges gave uniform recoveries for most of the components (60 to 80%) except for the highly polar metabolites such as ECG (less than 10%). Recoveries from the cyano and mixed-mode cartridges were generally poor.

Table 3
The % recovery±standard deviations of cocaine and its metabolites using an Alltech extra-clean silica SPE cartridge from pregnant rat blood, amniotic fluid, placenta and fetus

Analyte	% Extraction efficiency of silica SPE columns (±SD)			
	Blood	Amniotic fluid	Placenta	Fetus
CE	99.99±1.00	68.21±1.02	48.08±6.48	50.56±4.86
D_3-COC	54.41±1.46	74.3±7.48	59.31±10.36	55.64±7.65
COC	61.34±2.93	76.22±3.67	54.11±10.15	51.24±5.11
NCE	74.13±1.05	74.84±4.67	31.65±5.32	42.36±3.50
NC	81.48±7.13	72.88±3.02	46.86±7.34	54.27±4.63
PHOCOC	75.06±0.41	77.13±3.54	66.00±4.62	61.33±1.25
MHOCOC	57.22±1.65	61.86±4.34	39.77±4.19	41.06±4.07
PHOBE	68.65±4.60	96.88±0.98	57.15±3.06	65.70±7.29
BE	102.41±4.62	117.59±3.21	69.34±3.26	86.57±7.48
BN	47.81±1.38	67.13±1.06	31.70±3.55	39.16±1.75
EEE	62.52±0.58	79.63±6.78	49.81±6.09	30.13±4.70
EME	97.00±2.62	65.08±3.58	62.37±11.25	42.81±6.89
ECG	64.98±0.97	67.37±1.15	37.31±2.84	81.02±8.36
AEME	91.42±4.19	110.48±6.46	83.90±2.29	84.75±7.78

ECG is a very abundant metabolite, however its determination is rarely reported in the literature because its high polarity makes it very difficult to recover. One study of ECG reports a recovery of only 40% and even this required the use of two SPE cartridges in sequence [46]. Two other papers report the determination of ECG in whole blood and urine using very tedious and time consuming derivatization procedures with no recovery data reported [55,56]. The use of silica SPE cartridges, while somewhat unconventional, provides excellent recoveries for ECG, while still allowing for the recovery of the remaining metabolites in a single extraction procedure. Fig. 2 shows the LC–MS–MS trace of an extract of the fetal homogenate spiked with cocaine and its metabolites (50 ng/ml). There was negligible matrix interference even at the limit of detection of the assay. The blanks of all the four matrices from the same rat and from different animals do not show any significant difference and have been found to show very low background noise. This may be attributed to the high selectivity of the tandem mass spectrometric detection, which shows high selectivity and hence lower background noise. The other factors that contribute to the high selectivity and the low noise may be the efficiency of the chromatographic separation and sample clean up. Silica does not work through a classical chromatographic retention mechanism as has been reported in HPLC studies using it as a packing material [57–59]. Silica works for this extraction due to its high affinity for proteins. The proteins from the sample are highly bound to the silica while the sample is only weakly associated with silica. The elution of the samples using a strong organic ensures that the proteins will not become soluble and allows for the elution of the analytes, which can then be further concentrated following evaporation. Co-eluted compounds from the biological matrix do not affect the ionization and thus quantitation because the peak intensity of the spiked fluids and tissue extracts do not differ substantially from plain water extracts. The analyte peak intensities were found to be reproducible after extraction on using cartridges within the same batch, between cartridges from different manufactured batches, on different days, and irrespective of the rat from which the blank biological matrix was obtained.

3.5. Precision and accuracy of the method

The run-to-run standard deviations of recoveries from the extraction method was recorded. The inter- and intra-day standard deviations were low and indicated that the extraction method was rugged over the time frame studied. The recoveries from different batches of Alltech silica cartridges showed that the batch did not significantly affect the extraction efficiency or the quality of the extract. The extraction method consistently produced extracts that showed unnoticeable levels of background chemical noise in the LC–MS–MS chromatogram.

Intra-day ($n=3$) and inter-day ($n=9$) precision and accuracy were calculated from standard curves constructed for all analytes from each of the four biological matrices studied. From pregnant rat blood, the inter- and intra-day precision and accuracy for all 13 components (unknown concentration: 1 ppm) were in the range of 1.41–8.70% (RSD) and 0.21–19.86% (error), respectively. From amniotic fluid, the intra-day ($n=3$) and inter-day ($n=9$) precision and accuracy for all 13 components (unknown concentration: 1 ppm) were in the range of 1.15–11.90% (RSD) and 0.33–19.33% (error), respectively. From placenta, the intra-day ($n=3$) and inter-day ($n=9$) precision and accuracy for all 13 components (unknown concentration: 1 ppm) were in the range of 2.76–21.44% (RSD) and 0.58–16.85% (error), respectively. From fetus, the intra-day ($n=3$) and inter-day ($n=9$) precision and accuracy for all 13 components (unknown concentration: 1 ppm) were in the range of 2.76–25.54% (RSD) and 0.51–19.61% (error), respectively. These intra- and inter-day precision and accuracy data are tabulated in Table 4.

3.6. Analysis of pregnant rat samples

The in vivo metabolism of cocaine in pregnant rats was studied by infusing cocaine intravenously (as described in Section 2.3). A pregnant rat generally has 8–15 pups in any single litter. Each fetus is contained within an individual placenta. This allows a single pup to be removed and amniotic fluid, placenta and whole fetus samples to be taken at a given time point without disturbing the remaining pups. Due to the physiology of the pregnant rat, this

Table 4
The inter- and intra-day precision (% RSD) and accuracy (% error) of cocaine and its metabolites out of (A) pregnant rat blood, (B) amniotic fluid, (C) placental homogenate and (D) fetal homogenate

Analyte	Intra-day			Inter-day		
	Concentration found (ppm)	RSD (%)	% Error	Concentration found (ppm)	RSD (%)	% Error
Pregnant rat blood						
Concentration added: 1.0 ppm						
CE	1.09±0.02	2.14	9.22	1.11±0.07	6.06	10.79
COC	0.93±0.05	5.13	7.20	0.93±0.03	2.80	7.31
NCE	0.88±0.03	2.92	11.71	0.94±0.02	1.87	6.34
NC	0.80±0.06	7.97	19.86	0.87±0.06	7.16	12.57
PHOCOC	0.99±0.03	2.71	1.18	1.05±0.03	1.52	4.88
MHOCOC	0.85±0.02	2.75	14.58	0.97±0.03	2.64	3.18
PHOBE	0.98±0.09	8.70	1.70	1.01±0.05	5.31	1.07
BE	0.83±0.06	6.81	16.58	0.87±0.03	3.43	12.73
BN	0.89±0.05	5.45	11.04	1.08±0.04	3.79	7.96
EEE	0.92±0.02	2.50	8.26	0.96±0.03	2.81	3.96
EME	1.16±0.04	3.10	15.80	1.12±0.03	2.90	12.06
ECG	0.84±0.03	3.69	16.46	1.00±0.05	5.12	0.21
AEME	1.00±0.07	6.61	0.38	1.02±0.01	1.41	1.58
Concentration added: 0.25 ppm						
CE	0.29±0.03	6.06	10.79	0.28±0.01	2.49	1.03
COC	0.24±0.01	4.57	5.77	0.21±0.02	9.39	16.84
NCE	0.29±0.01	5.06	14.83	0.27±0.01	4.48	9.74
NC	0.29±0.01	2.82	17.67	0.29±0.03	8.60	16.44
PHOCOC	0.30±0.01	1.29	20.76	0.28±0.01	4.87	12.14
MHOCOC	0.22±0.01	0.15	13.83	0.22±0.01	6.76	13.12
PHOBE	0.34±0.01	4.33	37.86	0.29±0.01	1.52	16.89
BE	0.29±0.02	7.49	16.46	0.30±0.01	2.09	19.05
BN	1.26±0.02	6.09	3.44	0.26±0.01	3.40	4.34
EEE	0.29±0.01	2.84	16.21	0.26±0.02	5.72	4.86
EME	0.25±0.01	3.74	0.97	0.26±0.02	8.62	16.91
ECG	0.29±0.02	8.23	14.65	0.30±0.01	3.07	19.84
AEME	0.26±0.01	5.39	3.24	0.30±0.01	1.17	19.14
Amniotic fluid						
Concentration added: 1 ppm						
CE	1.19±0.07	5.89	19.33	1.06±0.05	4.42	6.08
COC	0.99±0.04	4.40	1.00	1.01±0.04	3.82	0.96
NCE	0.96±0.07	7.51	4.00	1.07±0.06	5.82	6.80
NC	0.80±0.06	11.90	3.77	1.02±0.03	2.55	2.06
PHOCOC	1.04±0.12	12.80	0.43	1.03±0.02	2.55	2.72
MHOCOC	1.00±0.13	1.15	0.33	0.97±0.03	1.30	0.41
PHOBE	1.00±0.01	9.54	0.33	1.00±0.01	4.69	6.35
BE	0.98±0.09	9.17	1.67	1.06±0.04	5.12	6.57
BN	1.08±0.13	11.72	8.27	1.07±0.06	5.22	7.28
EEE	0.97±0.05	4.72	3.37	0.99±0.01	1.38	0.90
EME	1.07±0.11	10.60	7.33	1.01±0.02	1.84	0.75
ECG	1.08±0.11	9.78	8.33	1.08±0.06	5.51	7.69
AEME	1.11±0.09	8.15	10.67	1.10±0.09	8.20	10.22

Table 4 (Continued)

Analyte	Intra-day			Inter-day		
	Concentration found (ppm)	RSD (%)	% Error	Concentration found (ppm)	RSD (%)	% Error
Concentration added: 0.25 ppm						
CE	0.24±0.04	15.02	4.00	0.23±0.007	2.87	9.10
COC	0.21±0.02	10.98	14.80	0.24±0.004	1.76	3.40
NCE	0.22±0.01	2.76	12.53	0.24±0.006	2.74	5.88
NC	0.30±0.01	2.56	19.33	0.26±0.003	1.03	3.70
PHOCOC	0.28±0.02	7.52	10.67	0.24±0.002	0.72	4.12
MHOCOC	0.21±0.01	5.41	14.67	0.22±0.003	1.56	12.13
PHOBE	0.30±0.02	6.29	18.13	0.22±0.007	3.04	12.91
BE	0.28±0.02	5.52	10.67	0.23±0.007	2.89	8.49
BN	0.26±0.01	5.27	5.33	0.19±0.005	2.69	24.80
EEE	0.24±0.03	12.01	4.93	0.28±0.010	3.67	9.93
EME	0.25±0.03	13.28	1.07	0.29±0.009	2.98	16.88
ECG	0.25±0.04	15.60	0.27	0.20±0.012	5.77	19.66
AEME	0.29±0.06	20.99	17.73	0.25±0.012	4.97	0.43
Placental homogenate						
Concentration added: 1.0 ppm						
CE	1.10±0.08	7.06	9.96	1.05±0.06	6.19	4.80
COC	1.05±0.03	2.76	5.22	1.11±0.03	2.99	10.71
NCE	1.01±0.08	8.18	1.08	1.12±0.24	21.44	11.98
NC	1.15±0.10	8.59	14.89	0.99±0.03	3.26	1.23
PHOCOC	1.25±0.35	27.89	24.89	1.10±0.18	15.94	9.90
MHOCOC	0.87±0.02	2.82	12.67	1.07±0.11	10.53	7.26
PHOBE	1.02±0.04	4.38	2.13	0.90±0.16	18.13	10.24
BE	0.99±0.13	12.90	0.76	0.83±0.15	17.92	16.85
BN	1.17±0.10	8.60	16.72	1.07±0.10	9.11	7.10
EEE	1.02±0.05	4.87	1.74	1.03±0.09	8.67	3.42
EME	0.99±0.06	5.65	0.58	1.97±0.17	17.07	3.17
ECG	1.08±0.05	4.77	8.44	1.10±0.15	13.88	10.03
AEME	1.14±0.13	11.20	14.19	0.93±0.18	19.25	6.90
Concentration added: 0.25 ppm						
CE	0.28±0.02	5.62	11.79	0.26±0.01	5.42	4.01
COC	0.24±0.01	1.38	5.68	0.21±0.02	7.99	16.30
NCE	0.21±0.01	2.16	17.42	0.25±0.01	3.38	0.14
NC	0.26±0.01	3.96	2.17	0.26±0.01	5.15	3.66
PHOCOC	0.24±0.01	4.18	3.37	0.25±0.02	9.69	0.45
MHOCOC	0.24±0.01	3.93	3.47	0.21±0.02	8.57	16.11
PHOBE	0.25±0.02	5.93	1.45	0.26±0.01	3.50	3.50
BE	0.29±0.03	9.07	14.10	0.25±0.01	2.59	1.76
BN	0.22±0.01	6.43	12.55	0.22±0.01	4.95	11.56
EEE	0.28±0.04	14.04	10.91	0.25±0.01	1.74	1.39
EME	0.26±0.04	13.37	5.55	0.21±0.01	5.54	16.99
ECG	0.26±0.03	10.57	4.91	0.29±0.02	8.29	15.86
AEME	0.30±0.01	2.43	18.11	0.30±0.01	3.77	19.93

Table 4 (Continued)

Analyte	Intra-day			Inter-day		
	Concentration found (ppm)	RSD (%)	% Error	Concentration found (ppm)	RSD (%)	% Error
Fetal homogenate						
Concentration added: 1.0 ppm						
CE	0.91±0.03	3.11	9.22	1.04±0.10	9.54	4.48
COC	0.92±0.01	0.48	7.75	1.17±0.08	6.97	16.59
NCE	0.88±0.03	3.71	12.15	1.07±0.10	9.44	6.64
NC	0.83±0.05	5.53	17.11	1.05±0.08	7.30	5.49
PHOCOC	0.84±0.05	5.88	15.75	1.13±0.18	15.69	13.26
MHOCOC	0.98±0.01	0.21	1.92	1.08±0.07	6.32	7.84
PHOBE	0.83±0.03	4.18	16.71	1.20±0.31	25.54	19.61
BE	0.84±0.06	7.51	16.30	1.07±0.07	6.57	6.94
BN	0.84±0.04	5.38	16.42	1.14±0.13	11.39	14.26
EEE	1.02±0.04	3.98	1.79	0.95±0.06	6.48	5.06
EME	0.91±0.01	0.74	8.77	0.93±0.06	6.64	7.38
ECG	0.98±0.08	7.69	2.25	1.03±0.10	9.32	2.73
AEME	0.81±0.2	2.19	18.83	1.01±0.18	18.36	0.51
Concentration added: 0.25 ppm						
CE	0.22±0.01	2.29	12.86	0.25±0.02	6.60	1.57
COC	0.23±0.01	0.68	7.31	0.30±0.01	2.93	18.72
NCE	0.24±0.01	2.41	5.79	0.22±0.01	6.50	11.83
NC	0.29±0.01	3.45	15.68	0.27±0.01	3.25	8.30
PHOCOC	0.27±0.01	3.16	7.25	0.28±0.04	13.21	11.74
MHOCOC	0.25±0.01	0.48	0.03	0.24±0.01	2.82	2.68
PHOBE	0.28±0.01	4.76	13.57	0.26±0.04	16.19	4.60
BE	0.29±0.01	3.62	17.23	0.28±0.02	7.45	10.21
BN	0.25±0.02	6.05	0.94	0.20±0.01	6.72	21.01
EEE	0.26±0.02	7.13	3.44	0.23±0.05	20.40	9.63
EME	0.20±0.03	13.89	19.65	0.26±0.05	20.43	5.22
ECG	0.30±0.01	4.61	19.64	0.23±0.03	14.81	6.10
AEME	0.25±0.01	2.51	1.38	0.22±0.02	7.36	10.75

model has an advantage over other animal models in that kinetic studies can be carried out using a single animal, thereby reducing variability.

Two pregnant rats were operated on two different days and each contained eight and 10 fetuses, respectively. The two rats provided multiple time points ($n=8$ and $n=10$) for the study of cocaine metabolism in their respective systems. The distribution of cocaine and its metabolites in the four compartments namely maternal blood, amniotic fluid, placenta and fetus were studied during steady state intravenous infusion of cocaine. When the maternal rats were exposed to cocaine, the drug transports from maternal blood through the placenta (where it may be retained and perhaps further metabolized) to the fetus and to the amniotic fluid. Thus, the method developed earlier could be utilized in studying the in vivo metabolism and cocaine distribution between the four compartments.

The results of the pregnant rat study were found comparable to the literature data thereby validating the reliability of our method for the study of cocaine biotransformation and distribution. Fig. 3 shows the extent of cocaine metabolism in pregnant rats in addition to the distribution of cocaine and its metabolites in the four compartments. The levels of cocaine and its metabolites in plasma, amniotic fluid, placenta and fetus in the two pregnant rats are shown in Table 5. Simone et al. reported the accumulation of cocaine in the placenta [16]. They also suggested that fetal exposure to cocaine and benzoylecgonine may be prolonged due to accumulation in the placenta, which would be undesirable [16]. In the first rat study, the accumulation of cocaine in the

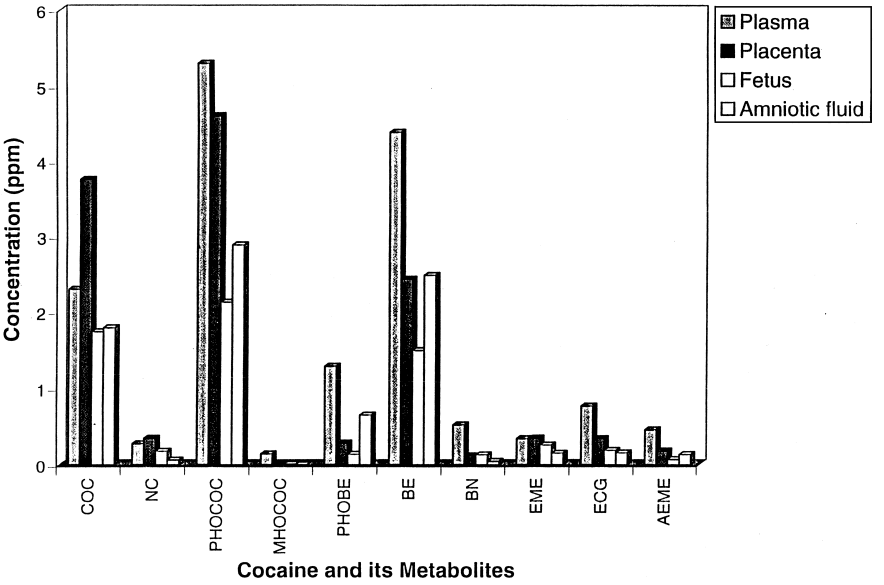

Fig. 3. The distribution of cocaine and its metabolites in maternal plasma, placenta, fetus and amniotic fluid in the pregnant rats on intravenous infusion of cocaine to a steady state plasma concentration.

placenta (concentration=3.17 ppm) was observed when compared to levels in the maternal blood (concentration=1.79 ppm). In the second rat, the level in placenta (concentration=4.36 ppm) and plasma (concentration=2.82 ppm) also indicated accumulation in the placenta, as observed by Simone et al. [16]. Metabolism of cocaine has also been observed in the placenta and the presence of esterases has been documented [21]. Previous studies have indicated that BE, EME and ECG are predominant pathways of cocaine biotransformation [44]. This was observed in the study of cocaine metabolism in the two pregnant rats (Fig. 3). In addition to the above, we observed that arylhydroxylation of cocaine was also a major metabolic pathway in pregnant rats. Levels of PHOCOC were found to be substantially higher than corresponding MHOCOC, the latter being found only in trace amounts. The fact that in humans the levels of the *para* and *meta* forms are comparable indicates interspecies variability (humans and rats) in phase I metabolism. This case illustrates the differences in the cytochrome P450 enzyme systems between humans and rats.

The accumulation of norcocaine was observed in the pregnant rats. Accumulation of this metabolite would be undesirable considering the possibility of the placenta acting as a reservoir for the sustained release of toxic norcocaine to the fetus. Substantial accumulation of cocaine and its major metabolites PHOCOC, PHOBE, BE, were also observed in the amniotic fluid. The study indicates that in the first rat, substantial maternal to fetal cocaine exposure (concentration in the fetus=1.54 ppm) takes place. Similar fetal cocaine exposures were also observed in the second pregnant rat (concentration in the fetus=1.95 ppm). The results also suggested that there was not a large difference in the cocaine metabolism and distribution between the various time points where sets of maternal blood, amniotic fluid, placenta and fetus were sampled within the same rat. The metabolism and distribution profiles of cocaine and its metabolites between the two rats were also observed to be similar. Thus, successful application of this bioanalytical LC–MS–MS method to the pregnant rat analysis of cocaine provides evidence of the applicability of the method for real time in vivo sample analysis. This study has helped

Table 5
The levels of cocaine and its metabolites in maternal plasma, placenta, fetus and amniotic fluid in the two pregnant rats on intravenous infusion of cocaine to a steady state plasma concentration

	COC (ppm)	NC (ppm)	PHOCOC (ppm)	MHOCOC (ppb)	PHOBE (ppm)	BE (ppm)	BNC (ppm)	EME (ppm)	ECG (ppm)	AEME (ppm)
Rat 1 (fetus=8)										
Plasma										
Mean concentration	1.79	0.06	2.33	8.36	0.56	1.62	0.10	0.20	0.51	0.16
Range of values	0.22	0.02	0.78	2.69	0.34	0.64	0.07	0.03	0.28	0.09
Amniotic fluid										
Mean concentration	1.78	0.03	1.54	ND	0.50	1.49	0.01	0.14	0.09	0.12
Range of values	0.19	0.01	0.38	ND*	0.17	0.36	0.01	0.03	0.03	0.02
Placenta										
Mean concentration	3.17	0.14	2.26	10.75	0.11	2.09	0.05	0.29	0.33	0.16
Range of values	0.25	0.02	0.47	3.64	0.03	0.53	0.02	0.05	0.08	0.02
Fetus										
Mean concentration	1.54	0.07	1.01	4.31	0.08	1.27	0.05	0.21	0.15	0.06
Range of values	0.12	0.02	0.23	1.38	0.04	0.44	0.02	0.04	0.08	0.01
Rat 2 (fetus=10)										
Plasma										
Mean concentration	2.82	0.50	8.28	280.42	2.03	7.17	0.96	0.49	1.04	0.77
Range of values	0.24	0.12	1.58	89.74	0.99	2.22	0.45	0.11	0.47	0.31
Amniotic fluid										
Mean concentration	1.78	0.09	4.08	ND	0.64	2.96	0.07	0.16	0.17	0.12
Range of values	0.48	0.04	1.44	ND	0.32	0.78	0.04	0.06	0.12	0.07
Placenta										
Mean concentration	4.36	0.57	6.97	35.48	0.47	2.78	0.19	0.42	0.36	0.21
Range of values	0.59	0.19	1.00	7.17	0.22	0.98	0.10	0.06	0.14	0.06
Fetus										
Mean concentration	1.95	0.29	3.25	10.19	0.20	1.74	0.22	0.32	0.23	0.08
Range of values	0.15	0.06	0.55	4.42	0.07	0.69	0.07	0.05	0.09	0.03

ND: Not detected.

in enhancing the current knowledge in this area by providing a holistic understanding of cocaine metabolism and distribution.

4. Conclusions

In this study, an efficient method was developed to simultaneously extract cocaine and its 12 metabolites from pregnant rat blood, amniotic fluid, placental and fetal tissues. We also report high recoveries for ecgonine using this simple extraction procedure. An LC–MS–MS method was developed and validated to analyze this complex mixture of drug and metabolites from four different biological matrices. Pregnant rats were infused with cocaine intravenously to a steady state plasma drug concentration and then the levels of cocaine and its metabolites were studied in the four biological matrices. The results of the pregnant rat study were comparable with the literature data thereby validating the reliability of our method for the study of cocaine biotransformation and distribution. The observations of accumulation of cocaine in the placenta suggesting protective effects to the fetus have been reported earlier [16]. We also support the premise that fetal exposure to cocaine may be prolonged due to accumulation of cocaine in the placenta [16]. The present study also

supports previous studies that indicate that BE, EME and ECG are predominant pathways of cocaine biotransformation. In the pregnant rat analysis, minor metabolic pathways were studied in addition to a comprehensive study of cocaine accumulation in the maternal and fetal compartments. This approach enhanced the current understanding of cocaine pharmacodynamics. The observations made are briefly summarized below. The arylhydroxylation of cocaine was identified as an additional major metabolic pathway in pregnant rats. *Para*-arylhydroxylation of cocaine is preferred over *meta*-arylhydroxylation in rats, unlike in humans. The accumulation of norcocaine in the placenta was also observed in the pregnant rats. Substantial accumulation of cocaine and its major metabolites PHOCOC, PHOBE, BE, were also observed in the amniotic fluid. Thus, the LC–MS–MS assay of cocaine and its metabolites from the four biological matrices aided in elucidating a more complete picture of cocaine metabolism, distribution and fetal exposure than currently available in the literature.

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